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► To cite this version:

Fanny Herisson, Marie-Françoise Heymann, Maud Chétiveaux, Céline Charrier, Séverine Battaglia, et al.. Carotid and femoral atherosclerotic plaques show different morphology.: Patterns of Peripheral Arterial Disease. *Atherosclerosis*, 2011, 216 (2), pp.348-54. 10.1016/j.atherosclerosis.2011.02.004 . inserm-00642808

HAL Id: inserm-00642808

<https://www.hal.inserm.fr/inserm-00642808>

Submitted on 18 Nov 2011

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Regular article

Carotid And Femoral Atherosclerotic Plaques Show Different Morphology

Short title: Patterns of Peripheral Arterial Disease

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Funding sources

This study was supported by grant from La Fondation de L'Avenir (ET7-477).

Total number of pages: 26

Total words count: 5292, included :

- Abstract :176
- Manuscript : 3566
- Figure legends :231
- Tables : 238
- References : 1081

Total number of figures and tables: 7

Abstract

Objective— Results of endovascular repair vary according to the arterial bed. We hypothesized that these differences may be related to the plaque features. To explore this hypothesis, we designed a prospective study that compared carotid and femoral atheroma.

Methods and Results— Patients that underwent femoral or carotid endarterectomy were included in our study. Demographic data and blood sampling were obtained prior to surgery. Plaques were evaluated for AHA grading, calcification and lipid content. Eighty eight plaques were harvested during this study (45 carotid specimens and 43 femoral specimens). No differences were noted between carotid and femoral groups regarding demographic and biological data. Histological data more frequently showed fibrous cap atheroma in carotid arteries (75%) and fibrocalcific plaques in femoral arteries (93%), $p<0.001$. Morphological analyses showed a high prevalence of osteoid metaplasia in femoral arteries (63 %) compared to carotid arteries (20%, $p<0.001$). Biochemical analyses were consistent with histological data, showing higher calcium and lesser cholesterol concentrations in femoral than in carotid plaques ($p<0.01$).

Conclusions— Femoral and carotid plaques showed different morphology in comparable groups of patients.

Keys words: peripheral artery disease, atherosclerosis, vascular calcification, lipids

Introduction

The definition of peripheral arterial disease (PAD) broadens towards a diverse group of disorders that lead to narrowing of supra-aortic trunks, aorta, upper and lower extremities arteries, and visceral arteries. PAD is a highly prevalent public health problem and is related to atherosclerosis. PAD has been classified as a coronary artery disease equivalent, meaning that patients with a diagnosis of PAD carry a risk for major coronary events equal to established coronary artery disease.¹ Given the high prevalence of coronary disease, research has focused for a long time on coronary arteries and little is known about the specificities of PAD.

Currently, more and more studies show different results after endovascular treatment of PAD according to the arterial bed. For instance, in-stent restenosis rates differ according to the arterial bed. In-stent restenosis following carotid stenting is reported to occur in less than 10% of cases², whereas it occurs in up to 40% of superficial femoral artery cases.³ In-stent restenosis following coronary stenting is reported to be in the range of 10% to 15%.⁴ However, stenting of lower extremities arteries is not as effective as stenting of coronary arteries since high rates of restenosis are observed in the range of 30% to 50%.⁵ Moreover, the efficacy of drug eluting stents (DES) differs according to the arterial bed. In 2001, Morice et al. reported 0% of coronary in-stent restenosis at 9 months.⁶ For peripheral arteries, DES have not shown to decrease in-stent restenosis in comparison to bare metal stents.⁷

Even if numerous factors such as the haemodynamic factors, the length of the lesion, the run-off could alter the restenosis rate, some studies have shown that the nature of the atheromatous plaque could be also a crucial factor of in-stent restenosis.^{8, 9} For example, different studies have observed by intravascular ultrasound that soft

atheromatous plaques are associated with a higher risk of in-stent restenosis.¹⁰

However, these studies did not identify the components of the plaque. Few studies have characterized atheromatous plaques according to the arterial bed. Some studies used non-invasive arterial assessment to evaluate calcification according to the arterial bed. CT scan for arterial calcium assessment is a non-invasive way of obtaining information about the presence, location and extent of calcified plaques in the arteries.^{11, 12} These studies have shown that calcification increases in older patients and varies according to each arterial bed. Furthermore, histopathological studies have noted the presence of osteoid metaplasia in a range of 9% to 13% of carotid plaques.¹³

To our knowledge, no previous study has compared advanced atherosclerotic lesions between different types of peripheral arteries. This study was designed to test the hypothesis of the existence of heterogeneity of atheroma expression among peripheral arteries such as carotid and femoral arteries.

Materials and methods

Patients

From February 2008 to June 2009, atheromatous plaques were harvested from patients undergoing carotid or femoral endarterectomy at the department of vascular surgery of the Nantes University Hospital. Patients suffering from non-atherosclerotic peripheral arterial disease, thrombosis or restenosis, and patients who could not give their written consent were excluded. Demographic and clinical data were collected, including age, gender, treatment, cardiovascular risk factors, the presence of coronary artery disease or renal failure was documented. Prior to surgery, blood specimens were collected for lipid balance and phospho-calcic metabolism. Sample collection and handling was performed in accordance with the guidelines of the Medical and Ethical Committee in Nantes, France, and a written informed consent was requested for each patient.

Tissue sampling

Endarteriectomies were performed on a consecutive series of patients using conventional surgical techniques. The plaque was removed at the bifurcation from within the lumen as a single specimen. All plaques were divided into 4 equal parallel sections. One part was processed for histological analysis, one section for scanning electron microscopy, and the last sections for lipid and calcium quantitative measurements.

Histology processing

Atherosclerotic plaques were immediately fixed in 10% formalin overnight and decalcified in Sakura TDE 30 fluid during 24 hours. They were embedded in paraffin.

Sections (4µm thickness) were stained with hematoxylin eosin (HE) added with safran. Lipid staining was obtained with Oil Red O for esterified lipids and filipin (filipin complex, F9765, Sigma-Aldrich, Poole, UK) for non-esterified lipids on frozen sections of 4µm as described by Kruth.¹⁴

Histological grade of the atherosclerotic plaque

The sections were graded according to a slightly modified AHA classification.¹⁵

Modified AHA classification is based on the 9 categories, of which 6 correspond to atheromatous plaques, type IX specimen (occlusion) were excluded from the study. Briefly these categories include fibrous cap atheroma (type IV), fibrous cap atheroma associated with fibrosis (type V), fibrocalcific plaque with more than 50% of plaque area calcified (type VII), fibrocalcific plaque that is mainly fibrous without a lipid core (type VIII). Complicated plaques (type VI) weren't taken into account since we were interested by rating the underlying plaque.^{15, 16} Inflammatory status of the plaque was determined semi quantitatively by the average number of macrophages stained by CD68 per mm² in the most representative part of the plaque and the percentage of CD68 stained area within the entire plaque.

Immunocytochemistry

Immunohistochemistry was performed on adjacent deparaffinized sections. Sections were incubated with 3% hydrogen peroxide to block endogenous peroxidase and the non specific bindings were blocked in 4% bovine serum albumin. Monoclonal antibodies directed against (1) CD68, a marker of macrophages (dilution 1/60; 2165 Immunotech, Marseille France); (2) CD31, a marker of endothelial cells (dilution 1/20; M823, Dako Ltd, Ely, UK); (3) smooth muscle-actin (prediluted, A2547, Sigma-Aldrich, Saint-Quentin Fallavier, France). Biotinylated polyclonal anti-mouse secondary

antibody and peroxidase-conjugated streptavidin were applied for 1 hour each with the use of the extravidine peroxidase for 30 min and then revealed with an AEC staining kit (Sigma Aldrich). Preparations were counterstained with HE. A negative control was analyzed using a similar procedure excluding the primary antibody.

Quantitative measurements of calcium and lipids within the plaque

Exact amounts of tissue samples, 30 to 50 mg each, were carefully weighted, 5- α cholestane added for internal marker, homogenized with chloroform/methanol (2:1), and the homogenate was extracted two times with the mixture. The extract was evaporated and was saponified 1h at 70°C. Sterols were extracted with cyclohexane. After evaporation, sterol fractions were silylated. Cholesterol was quantitated with gas chromatography-mass spectrometry (GC-MS). The values are given as mg/g of tissue. For analysis of calcium, 50 samples were first dehydrated. After carbonization (550°C, 12 hours) ashes were dissolved in 1 ml of nitric acid and then 24 ml of 1% lanthanum oxide (LaCl₃). Calcium absorbance was then determined by atomic absorption spectroscopy at 422.7 nm using a calcium lamp (Unicam Solaar 989 Atomic Absorption Spectrometer, Cambridge, UK) and compared to a standard curve constructed using known dilutions of a solution of 1000 μ g/ml Ca in 1% LaCl₃. Quantitative measurements were expressed in milligrams for gram of dried tissue. For some samples the length of the arterial segment available was not sufficient to perform all analyses, such specimens did not represent specific subgroup analysis.

Scanning electron microscopy (SEM)

GMA-embedded plaques (10 carotid and 8 femoral specimens) were polished with graded silicon carbide grinding paper (Struers Denmark) and then gold-palladium-

coated on a Desk III (Denton Vacuum, Moorestown, USA). SEM studies were performed with backscattered electrons (Leo 1450 VP, Zeiss, Oberkochen, Germany). Phospho-calcic ratios were determined by energy dispersive system X ray analysis (Inca Oxford instrument, UK).

Statistical analysis

Statistical analysis was performed using SPSS 10.0 software. Comparison of the data was assessed by either the Chi Square test or the two-sample Student's t-test. For phospho-calcic ratio analysis, non-parametric the Mann and Whitney test was used. $P < 0.05$ was considered significant.

Results

Baseline characteristics

Eighty eight patients were enrolled in the study; 45 underwent carotid endarterectomy (mean age 69.7 ± 1.65 years, range 36-84) and 43 underwent femoral endarterectomy (mean age 69.2 ± 1.5 years, range 59-85). In carotid endarterectomy specimens, 7 and 38 patients were respectively symptomatic and asymptomatic. The clinical presentation of symptomatic patients was detailed in table 1. A comparison of baseline clinical and biological preoperative data is shown in table 2. There was no significant difference between the two groups in terms of age, gender, cardiovascular risk factors, coronaropathy disease, renal failure, statins, antiplatelet therapies and vitamin K antagonists ($p=NS$). Lipid and phosphocalcic parameters were similar in both groups of patients, regardless of vitamin D level, which was lower in patients with femoral endarterectomy ($p<0.05$).

AHA specimen classification

The histological analysis according to AHA modified classification for human femoral and carotid plaques is shown in Figure 1. Seventy five percent of carotid plaques were classified as fibrous cap atheroma (types IV and V) *versus* 7% of the femoral plaques ($p<0.001$). On the other hand, 93% of femoral plaques were classified as fibrocalcific plaques (types VII and VIII) *versus* 25% of carotid plaques ($p<0.001$).

Femoral and carotid plaques display different mineral patterns

Sampling of human atherosclerotic plaques from patients undergoing carotid or femoral endarterectomy indicated the presence of 4 types of calcifications: sheetlike calcification, nodular calcification, clear centre calcification and osteoid metaplasia. Sheetlike calcification was defined as a calcification front within fibrosis, surrounded by numerous calcified micronodules (Figure 2A and 2E). Nodular calcification was characterized by numerous stratified deposits of calcification with multinodular edges, consistent with an aggregation phenomenon, and the presence of very few cells (Figure 2B and 2F). Clear centre calcification consisted of vesicle- like structure outlined with calcium deposits (Figure 2C and 2G). The last type of calcification was osteoid metaplasia that consisted of mature bone with typical lamellar structure and often bone marrow (Figure 2D and 2H). The prevalence of each type of calcification was compared (Figure 3A). Femoral plaques exhibited significantly more sheetlike calcification (86% vs 62%; $p<0.05$) and nodular calcification (84% vs 58%, $p<0.01$) when compared to carotid plaques. The clear centre calcification prevalence was similar between both specimen types. Femoral plaques more often displayed osteoid metaplasia (63% vs 20% $p <0.001$) when compared to carotid plaques. We noticed that osteoid metaplasia always developed in deep regions of the intimal layer, close to the media and vasa vasorum (data not shown). Histological analysis of sampling of femoral and carotid plaques did not indicate higher density of calcium in the outer part of the media. Twenty seven carotid specimens and 23 femoral specimens were analyzed to determine the calcium level within the plaque. Femoral plaques displayed significantly higher amounts of calcium (194 ± 33 mg/g vs 85 ± 16.2 mg/g; $p<0.01$) as compared to carotid plaques (Figure 3B). The phospho-calcic atomic ratio determined by SEM was similar in carotid (1.89 ± 0.05) and femoral plaques ($1.85 \pm$

0.34), $p=0.11$. These results are consistent with the presence of hydroxyapatite cristal.

CD31 and smooth muscle-actin immunostaining were not different between atherosclerotic plaques from patients undergoing carotid or femoral endarterectomy.

Carotid plaques display more lipid and inflammation patterns than femoral plaques

We observed intra- and extracellular lipid deposits within the plaque. Non esterified cholesterol was found within the macrophages (Figure 4 A). Results for quantitative analyses are shown in Figure 4B. Total cholesterol values differed significantly between carotid and femoral plaques. Mean cholesterol levels were higher in carotid plaques compared to femoral plaques (39.85 ± 5.6 mg/g vs. 10.5 ± 1.79 mg/g; $p < 0.001$). Concerning the distribution of inflammatory cells, twenty carotid and femoral specimen were analyzed. The mean total percentage of macrophage area was $15.2 \pm 3.14\%$ within carotid plaques versus $6.13 \pm 2.2\%$ within femoral plaques ($p < 0.05$) (Figure 5A). Carotid plaques (figure 5C) showed a higher content of CD68 positive cells in relation to the total section area than femoral plaques (figure 5D): 310.5 ± 49.15 versus 174.5 ± 39.75 / mm^2 , $p < 0.05$, (Figure 5B).

Discussion

In this study we have observed that, in comparable groups of patients, femoral and carotid plaques showed different morphology. Indeed, carotid arteries displayed more lipid and inflammatory content than femoral arteries while femoral arteries were more prone to calcify and to develop osteoid metaplasia.

Site specificity for atherosclerosis is largely described. Numerous factors could influence regionally distinct atherosclerotic lesion development and therefore morphology of atheromatous plaques. Site-selectivity of atherosclerotic lesions includes differences in hemodynamics, the underlying wall structure, cardio-vascular risk factors and cellular or biochemical parameters of the arterial wall. Site-specific arterial wall structure could have a potential influence. However, carotid and femoral artery bifurcations are both considered as a transitional zone between elastic and muscular artery types without striking arterial geometry differences.¹⁷ Regarding blood flow patterns, this varies in different regions in quantitative detail and these variations could have a complex effect on the development of atherosclerosis. For example, peak/mean wall shear rates are higher in carotid arteries than in femoral artery.¹⁸ Blood flow variations may influence the relative residence time of lipoproteins, blood borne molecules and inflammatory cells that come in contact with the endothelial cells in each of these regions.¹⁹ On the other hand, the hemodynamic patterns may prime the gene expression profile of endothelial cells in subtle different ways so that these cells react differently to cardiovascular risk factors.²⁰ Like coronary artery lesions, PAD is influenced by cardio-vascular risk factors. For instance, Baumgartner et al. have described a different distribution pattern of lower

limb atherosclerosis according to the cardiovascular risk factor profile of patients with peripheral arterial occlusive disease.²¹ Risk factor profiles for each vascular territory are different insofar as there is some evidence of a stronger association between large vessel disease and smoking and dyslipidemia, whereas diabetes appears more specific for small vessel disease. In their study, patterns of plaques were not described. Different studies have already reported cellular or biochemical variations in the arterial wall. To test the hypothesis of intrinsic differences in the artery wall, Haimovici et al. used aortic homograft transplantation to examine the responses of different aortic segments to a high fat atherogenic diet. Aortic segments in dogs from atherosclerosis-resistant thoracic aorta were transplanted into atherosclerosis-susceptible abdominal aorta and vice versa. After 1 year, the authors observed that atherosclerosis-resistant segments transplanted into atherosclerosis-prone positions in the abdominal aorta remained lesion free even though abdominal aorta flanking the transplanted thoracic segment developed severe aortic atherosclerosis. In the same animals, atherosclerosis-prone segments transplanted into atherosclerosis-resistant positions still developed severe aortic atherosclerosis even though the flanking thoracic aorta was lesion-free.²² The diversity of the origins of vascular cells could provide explanations about how blood vessels differ from one another and why they respond in disparate ways to common risk factors associated with vascular disease. Indeed, Majesky emphasizes that smooth muscle cells arise from distinct sources of progenitors, each with its own unique lineage and developmental history and consequently participate to the vascular bed-specific patterns of atherosclerosis.²³

In the present study, the calcium content was higher in femoral plaques than in carotid plaques although the phospho-calcic atomic ratio was similar between both types of plaques. There is no evidence for higher prevalence of Monckeberg's disease in femoral versus carotid specimens in our study. Nevertheless, Mönckeberg's sclerosis is common in peripheral artery it could be difficult to distinguish Monckeberg's lesions from advanced atherosclerotic lesions.²⁴ CT scan for arterial calcium is a non-invasive tool for obtaining information about the presence, location and extent of calcified plaques in the arteries.¹¹ The findings on CT scans are expressed as a calcium score. These studies have shown that calcification varies according to each arterial bed. For example, calcium scoring was higher in iliac arteries than in carotid arteries. Differences in the content of calcium according to the arterial region is consistent with the histological grading that showed that femoral plaques are classified as fibrocalcific (VII and VIII types) whereas carotid plaques are classified as fibrous cap atheroma (IV and V types). In an autopsy study, Dalager et al. analyzed the coronary, carotid and superficial femoral arteries from 100 individuals.¹⁵ They clearly showed the artery related differences in phenotypic expression of atherosclerotic plaques. In the bifurcated carotid region most of the plaques were lipid core plaques (types IV and V) and more than half of femoral plaques were classified as fibrous type VIII lesions. In another autopsic study, Sawabe et al revealed that the severity of atherosclerosis differed among arteries. They observed that the aorta and arteries of the lower extremities were severely affected, while the abdominal arteries, such as the splenic and superior mesenteric arteries, were mildly affected.²⁵ Initiation and rate of lesion progression should be taken into account to interpret the histological grading related differences. Indeed our analysis is confined to a single point and assumes a similar rate of atheromatous

1 disease progression in each plaque location. Moreover, Stary showed that the
2 natural trend of the plaque was toward a fibrocalcific phenotype.²⁶ According to
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4 Dalager et al., carotid plaques seem to be observed sooner than femoral plaques
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6 with a prevalence of lipid core plaques.¹⁵ Consequently, these findings suggest the
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8 hypothesis of different kinetics of progression of atheromatous plaques according to
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10 the arterial region, however to date no data are available on this matter.
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15 Femoral plaques exhibit significantly more sheetlike calcification, nodular calcification
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17 and osteoid metaplasia than carotid plaques. The most striking difference between
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19 both types of plaques is related to the prevalence of osteoid metaplasia in femoral
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21 plaques. Up to date, different studies reported the presence of both cartilaginous and
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23 bone formation in atheromatous plaques but few studies reported prevalence of
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25 osteoid metaplasia in peripheral arteries.^{13, 27, 28} In carotid arteries, the prevalence of
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27 ossification was evaluated approximatively between 9% to 13%.^{13, 28} These data are
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29 consistent with our own results. The origin of this pathological change has been
30
31 attributed to metaplastic osteogenesis along an endochondral pathway.²⁹ Although
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33 we have punctually found cartilage tissue in our specimens (data not shown),
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35 membranous ossification could not be excluded. In membranous ossification bone is
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37 built via the differentiation of mesenchymal cells in osteoblasts that product osteoid
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39 matrix that is further mineralized.
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48 We observed a significant increase of the serum level of vitamin D in the patients
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50 from the carotid group although all levels were clearly under normal values in both
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52 groups. It is clear that vitamin D could influence PAD because of its effect on target
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54 cells and tissues.³⁰ However, there is controversy concerning the action of vitamin D
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56 on arterial calcification in humans. Even though vitamin D promotes calcifications in
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vitro³¹, in clinical studies authors reported either no effect or an inverse relationship between vitamin D levels and vascular calcification.³²⁻³⁴

The present study is a cross-sectional study of carotid and femoral plaques obtained from different patients at a single point in time. Consequently, we cannot rule out a bias created by harvesting carotid and femoral plaques in different patients. Indeed, in order to obtain a better matching, carotid and femoral plaques should be harvested in the same patient. Obviously, few patients require both femoral and carotid endarterectomy at a single point in time. Although we can only postulate that the observed lesions represent the late plaque lesion, further studies of plaques are needed with a wide range of disease severity to further define plaque morphology, cellular and molecular factors involved in progression from the apparent early lesion to clinically significant artery stenosis. Furthermore, factors such as the delay between symptoms onset could influence plaque type. These factors differ between groups and could be a limit to this study. In carotid arteries, surgery is requested for different degree of stenosis according the symptomatology.³⁵ Moreover, in symptomatic carotids, it is recommended to undergo surgery in the 15 days following the onset of symptoms.³⁵ For femoral arteries, each patient underwent conservative therapy combined at least for 3 months before surgical procedure but the delay prior to surgery differs a lot between each patient according their clinical history.

The results of this study demonstrate that femoral and carotid plaques show different morphology at a single point in time. Mechanisms and clinical implications of atheroma plaques heterogeneity within the arterial tree should be investigated for a better understanding of the characteristics of the arteries and to choose the best medical, endovascular or surgical option to treat atheromatous lesions.

Acknowledgments

We thank Jean-Baptiste Michel, Béatrice Guyomarch, Alexandra Auvrignon, Karine Fajoles, Brigitte Hauraix and Stéphanie Crossouard for their excellent assistance. We also thank Marja Steenman for reviewing the English text of this manuscript.

Disclosures

None

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Figure legends

Figure 1 : Histological classification of carotid and femoral plaques according to a slightly modified AHA classification (15). Type IV and V are defined as fibrous cap atheroma. Type VII and VIII are defined as fibrocalcific plaques.

Figure 2: Hematoxylin eosin and safran (HES) staining and scanning electron microscopy (SEM) backscattered electron images. (A) HES stain, original magnification X200 , sheetlike calcifications. (B) HES stain, original magnification X200, nodular calcifications. (C) HES stain, original magnification X400, clear centre calcifications. (D) HES stain, original magnification X100, osteoid metaplasia. (E) SEM, original magnification X 1000, sheetlike calcifications (F) SEM, original magnification X 500, nodular calcifications (G) SEM, original magnification X 1000, clear centre calcifications (H) SEM, original magnification X 250, osteoid metaplasia.

Figure 3: Qualitative (A) and quantitative (B, expressed as mean \pm SEM) assessments of mineral patterns in carotid and femoral plaques. *P< 0.05, **P< 0.01, ***P< 0.001

Figure 4: (A) Lipid staining was obtained with filipin and macrophages were stained by a monoclonal antibody directed against CD68. (B) Quantitative measurements of

cholesterol within carotid and femoral plaques were expressed in milligrams per gram
± SEM of dried tissue. **P<0.01

Figure 5: Quantitative measurements of macrophages within carotid and femoral
arteries (A,B) * P< 0.05. C : Example of CD68 staining of a carotid specimen,
original magnification X50. D : Example of CD68 staining of a femoral specimen,
original magnification X50.

Figure1



Figure 2

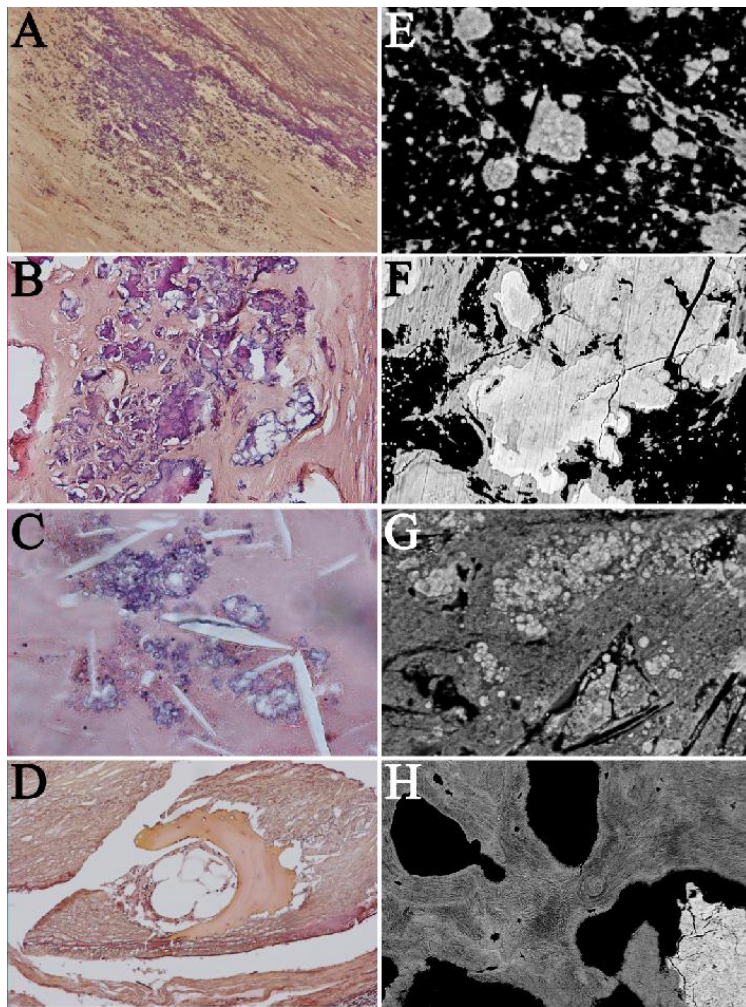


Figure 3

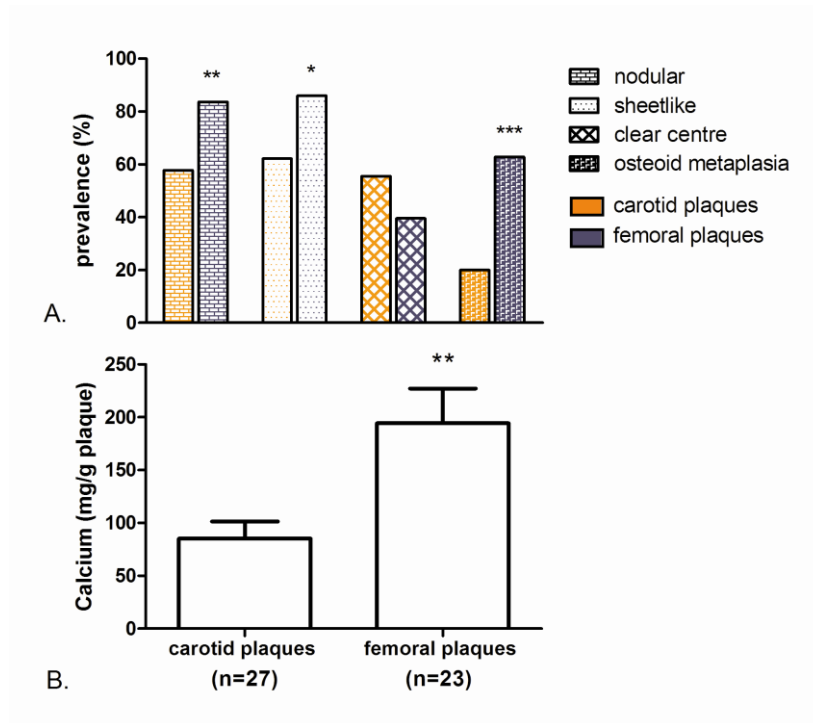


Figure 4

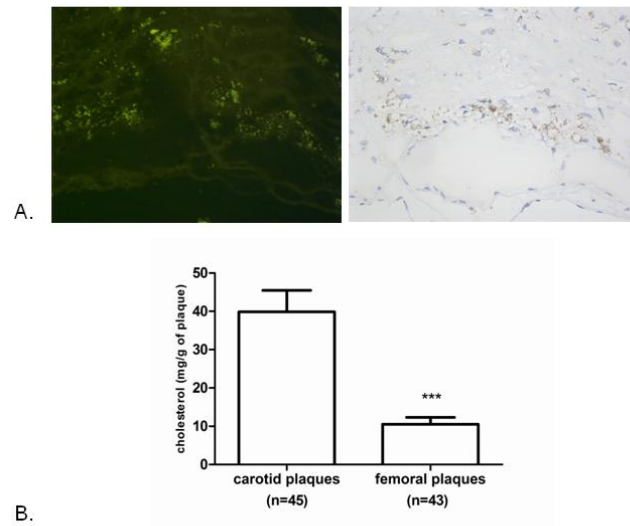


Figure 5

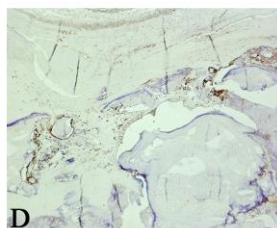
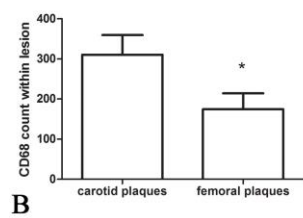
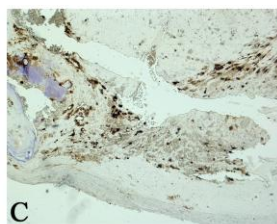
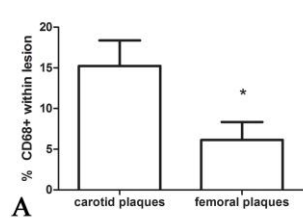


Table 1: Clinical presentation and delay to surgery of the seven symptomatic carotid patients.

SYMPTOMS	TYPE	DELAY TO SURGERY (days)
Hemiparesis	infarction	147
Aphasia and brachiofacial paresis	infarction	118
Brachiofacial paresis	infarction	58
Facial paresis	infarction	40
Amaurosis fugax	transient ischemic attack	120
Brachiofacial paresis	infarction	15
Aphasia and brachiofacial paresis	infarction	15

Table 2: Baseline characteristics, treatment and biomarkers according to atheromatous plaque location

	Carotid plaques (n=45)	Femoral plaques (n=43)	p
Demographic data and medical History*			
Age, y	69,7 (± 1.65)	69,2 (± 1.5)	0.8
Male, % (n)	71,1 (32)	86 (37)	0.12
Hypertension, % (n)	88.9 (40)	81.4 (35)	0.32
Diabetes, % (n)	17.8 (8)	20.9 (9)	0.71
Hypercholesterolaemia, % (n)	82.2 (37)	83.7 (36)	0.85
Smoking, % (n)	71.1 (32)	72.1 (31)	0.92
Body mass index, kg/m2	26.5 (± 0.7)	26.4 (± 0.6)	0.92
Coronaropathy, % (n)	55.6 (25)	53.5 (23)	0.85
Renal failure, % (n)	48.9 (22)	41.9 (18)	0.51
Medications*			
Statins, % (n)	84.4 (38)	76.7 (33)	0.36
Vitamin K antagonists, % (n)	2.2 (1)	4 (9.3)	0.15
Antiplatelet agents, % (n)	100 (45)	93 (40)	0.11

Biomarkers*

LDL-C (g/L)	1 (± 0.05)	0.94 (± 0.05)	0.36
Vitamin D (ng/mL),	17.7 (± 1.58)	12,26 (± 1.06)	0.006
PTH (pg/mL)	51.96 (± 4,26)	54 (± 3.55)	0.71
Ionised calcium (mmol/L)	1.24 (± 0.01)	1.22 (± 0.01)	0.3

*Clinical characteristics 'medications' and 'biomarkers' are expressed as mean±SEM or % (n). LDL-C = low-density lipoprotein cholesterol